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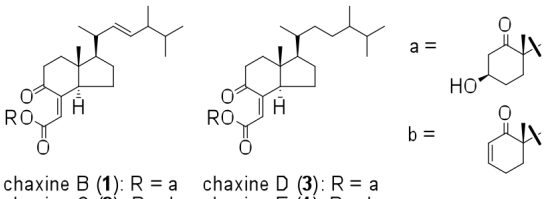

chaxine B (**1**): R = a chaxine D (**3**): R = a
chaxine C (**2**): R = b chaxine E (**4**): R = b

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ABSTRACT

Four novel compounds, chaxines B (**1**), C (**2**), D (**3**) and E (**4**) were isolated from an edible mushroom *Agrocybe chaxingu*. The structures of **1**-**4** were determined by the interpretation of spectroscopic data. Compounds **1** and **2** suppressed the formation of osteoclast.

1. Introduction

Bone homeostasis during remodeling is maintained by osteoclastic bone resorption and osteoblastic bone formation[1]. Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage family. They are the principal, if not exclusive, resorptive cell of bone, playing a central role in the formation of the skeleton and regulation of its mass. Boneforming cells, or osteoblasts, have an equally important role in the regulation of bone mass[2]. Osteoporosis is caused by an imbalance between bone resorption and bone formation, which results in bone loss and fractures after mineral flux. The hip fracture in senile patients is a very serious problem because it often limits their quality of life. Recently, beneficial effects of natural products and their derivatives that affect the process of bone remodeling, in particular bone resorption, have been reported. For example, reveromycin A is a promising agent that inhibits bone resorption by specific induction of apoptosis in osteoclasts[3]. Chaxine A has been isolated as an osteoclast-forming suppressing compound from the edible mushroom *Agrocybe chaxingu* (Agaricomycetideae)[4]. Two sterols also have been reported as the suppressive compounds from *Zizania latifolia* infected with *Ustilago esculenta* (Makomotake in Japanese)[5]. Osteoclast-like multinucleated cells can be differentiated *in vitro* from co-cultures of mouse bone marrow cells and osteoblastic cells by treatment with osteotropic factors, 1α,25-dihydroxyvitamin D_3 and prostaglandin E2. During screening for the osteoclast-formation suppressing effects of the extracts of various mushrooms by using the assay, we found strong activity in the extract of the mushroom *A. chaxingu*, and tried to isolate the active principles from the mushroom. This mushroom grows in dry and died boles of broadleaf such as grease tea

plants and poplar, and exists only in mountainous areas in South China. Here we describe the isolation, structural determination, and biological activity of four novel compounds from the mushroom.

2. Results and discussion

The dried fruiting bodies of *A. chaxingu* were extracted with CH_2Cl_2, EtOAc and then EtOH. Since only the CH_2Cl_2 soluble fraction showed the suppressing activity against the formation of osteoclast, this fraction was repeatedly subjected to column chromatography, being guided by the result of the bioassay. As a consequence, four novel compounds (**1**-**4**) were purified.

Chaxine B (**1**) was purified as colorless oil. Its molecular formula was determined as $C_{28}H_{42}O_5$ by HRESIMS *m/z* 481.2946 $[M+Na]^+$ (calcd for $C_{28}H_{42}NaO_5$, 481.2930). The structure of **1** was elucidated by interpretation of NMR spectra including DEPT, COSY, HMBC, and HMQC (Fig. 1). The complete assignment of the protons and carbons was accomplished as shown in Table 1. The indene skeleton was elucidated by the COSY correlations (H5/H6, H8/H9, H10/H11) and the HMBC correlations (H5α/C3, H5α/C4, H5α/C6, H5α/C7, H5β/C4, H5β/C6, H6α/C5, H6α/C7, H6β/C4, H6β/C5, H6β/C7, H6β/C8, H8/C3, H8/C4, H8/C6, H8/C7, H8/C9, H9/C7, H9/C8, H9/C10, H9/C11, H10/C7, H10/C8, H10/C9, H10/C11, H11/C6, H11/C7, H11/C9, H11/C10). The structure of the side chain was determined by the COSY correlations (H20/H18, H19/H18, H18/H17, H17/H21, H17/H16, H15/H16, H13/H14, H13/H15). The linkage between C11 and C13 was elucidated by the COSY and HMBC correlations (H11/H13; H10/C13, H11/C13, H11/C14, H13/C11, H14/C11, H15/C11). The HMBC cross peaks

(H6/C12, H8/C12, H11/C12, H12/C6, H12/C7, H12/C8, H12/C11) indicated the bond between C12 and C7. The HMBC correlations (H2/C1, H2/C3, H2/C4, H2/C8, H8/C2), and the chemical shifts of C1 (δ_C 165.3) and C4 (δ_C 203.5) suggested the presence of γ-keto-α,β-unsaturated carboxyl moiety in **1**. The partial structure of **1** (C1 to C21) is corresponding to the acid form of **5**, which has been isolated from this mushroom[4]. The 2,5-dihydroxy-2-methylcyclohexanone moiety was constructed by the COSY correlations (H4′/H3′, H4′/H5′, H5′/H6′) and the HMBC correlations (H3′/C1′, H3′/C2′, H3′/C4′, H3′/C5′, H4′/C2′, H4′/C3′, H4′/C5′, H4′/C6′, H6′/C1′, H6′/C2′, H6′/C4′, H6′/C5′, H7′/C1′, H7′/C2′, H7′/C3′). The ester bond between the indene skeleton (C1 to C21) and the cyclohexanone moiety (C1' to C7') was determined by the lower downfield chemical shift of C2′ (δ_C 82.6) and the molecular formula of this compound. As a result, the plane structure of **1** was determined as shown. The relative stereochemistry of the alcohol and acid parts in **1** was determined by NOE difference and/or NOESY experiments, respectively (Fig. 1). However, the absolute configuration of both the parts remains undetermined.

Chaxine C (**2**) was isolated as colorless oil. Its molecular formula was determined as $C_{28}H_{40}O_4$ by HRESIMS *m/z* 463.2845 $[M+Na]^+$ (calcd for $C_{28}H_{40}NaO_4$, 463.2824). The NMR data of **2** were very similar to those of **1** (Table 1). Comparison of the molecular formula of **2** with that of **1** indicates that **2** is a dehydrated form of **1**. The position of the dehaydration was elucidated by chemical shifts of position 5′ (δ_H 6.86, δ_C 148.1) and 6′ (δ_H 6.01, δ_C 128.1), and the other NMR data. As a result, the structure of **2** was determined as shown. The relative stereochemistry of **2** was the same as that of **1** and its absolute configuration remains undetermined.

Chaxine D (**3**) was purified as colorless oil. Its molecular formula was determined as $C_{28}H_{40}O_5$ by HRESIMS *m/z* 483.3062 $[M+Na]^+$ (calcd for $C_{28}H_{44}NaO_5$, 483.3086). The ^1H and ^{13}C NMR data of **3** were very similar to those of **1** (Table 1). However, **3** has two sp^3 carbons instead of an olefin in the side chain of **1**. Chaxine E (**4**) was isolated as colorless oil. Its molecular formula was determined as $C_{28}H_{38}O_4$ by HRESIMS *m/z* 465.2980 $[M+Na]^+$ (calcd for $C_{28}H_{42}NaO_4$, 465.2980). The ^1H and ^{13}C NMR data of **4** were very similar to those of **2** and **3** (Table 1). As a result, the structures of **3** and **4** including their relative stereochemistry were determined as shown.

Compounds **1** and **2** were evaluated in the osteoclast-forming assay (Fig. 2). The assay is based on the principle that osteoclast-like multinucleated cells can be formed *in vitro* from co-cultures of mouse bone marrow cells and osteoblastic cells by treatment with osteotropic factors. By adding suppressive agents, the formation of osteoclast is inhibited during the differentiation. As shown in Fig. 2, **1** and **2** at 3.1 μg/mL suppressed significantly the rate of osteoclast formation to 66% and 0% with no cytotoxicity, respectively. We have also previously isolated **5** and **6** from this mushroom as the osteoclast-forming suppressive substances[4]. Based on our results, this mushroom shows the potential of forming a supplement to the staple diet as a functional food to improve and/or prevent osteoporosis. However, the detailed mechanism of the effects of the compounds remains unsolved.

3. Experimental

3.1. General

^{1}H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. The HRESIMS spectra were measured on a JMS-T100LC mass spectrometer. A JASCO grating infrared spectrophotometer was used to record the IR spectra. The specific rotation values were measured by using a JASCO DIP-1000 polarimeter. HPLC separations were performed with a JASCO Gulliver system using a reverse-phase HPLC column (Wakopak Navi C30-5, Wako, Japan). Silica gel plate (Merck F_{254}) and silica gel 60 N (Merck 100-200 mesh) were used for analytical TLC and for flash column chromatography, respectively.

3.2. Fungus materials

Mature fruiting bodies of *A. chaxingu* were collected in Fujian Sheng, China, and identified by M. Takahashi of Kougen Co. Ltd. Voucher specimens were deposited in the Faculty of Agriculture, Shizuoka University.

3.3. Extraction and Isolation

Powder of the dried fruiting bodies of *A. chaxingu* (1.5 kg) was successively extracted with CH_2Cl_2 (3 L, 2 times), EtOAc (3 L, 2 times) and then EtOH (3 L, 2 times). The CH_2Cl_2-soluble part (32.4 g) was fractionated by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 95:5, 9:1; CH_2Cl_2/EtOH 9:1; and EtOH, 1.2 L each) to obtain 17 fractions, and fraction 5 (1.3 g) was further separated by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 95:5, 9:1; acetone; and EtOH, 2 L each) and 8 fractions were obtained. Fraction 5-5 (191.3 mg) was further separated by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 99:1; and EtOH, 2 L

each) affording 8 fractions. Fraction 5-5-6 (21.4 mg) was further separated by reverse-phase HPLC (Wakopak NaviC30, 90% MeOH) to afford compounds **1** (2.1 mg) and **3** (0.5 mg). Compounds **2** (1.6 mg) and **4** (0.4 mg) were obtained from fraction 5-5-2 (7.2 mg) by reverse-phase HPLC (Wakopak NaviC30, 85% MeOH).

3.3.1. Chaxine B (**1**)

Colorless oil; $[\alpha]_D^{27}$ +60 (*c* 0.2, $CHCl_3$); IR (neat): 3431, 1729, 1644 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m/z* 481 $[M+Na]^+$; HRESIMS *m/z* 481.2946 $[M+Na]^+$ (calcd for $C_{28}H_{42}NaO_5$, 481.2930).

3.3.2. Chaxine C (**2**)

Colorless oil; $[\alpha]_D^{26}$ +46 (*c* 0.2, $CHCl_3$); IR (neat): 1734, 1680, 1620 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m/z* 463 $[M+Na]^+$; HRESIMS *m/z* 463.2845 $[M+Na]^+$ (calcd for $C_{28}H_{40}NaO_4$, 463.2824).

3.3.3. Chaxine D (**3**)

Colorless oil; IR (neat): 3405, 1729, 1642 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m/z* 483 $[M+Na]^+$; HRESIMS *m/z* 483.3062 $[M+Na]^+$ (calcd for $C_{28}H_{44}NaO_5$, 483.3086).

3.3.4. Chaxine E (**4**)

Colorless oil; IR (neat): 1734, 1680, 1653 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m/z* 465 $[M+Na]^+$; HRESIMS *m/z* 465.2980 $[M+Na]^+$ (calcd for $C_{28}H_{42}NaO_4$, 465.2980).

3.4. Bioassay

The stromal/osteoblastic cells, UAMS-32, were cultured in an α-minimal essential medium (α-MEM) (ICN Biomedicals, Inc.) containing 10% fetal bovine serum (FBS) for a week. The cells were detached from the culture dishes by using trypsin-EDTA, suspended in α-MEM containing 10% FBS and used for the co-culture as osteoblastic cells. Bone marrow cells were isolated from mice as described previously[6]. Femoral and tibiae bone marrow cells were collected from 5-week-old mice which had been killed by cervical dislocation. The tibiae and femora were removed and dissected free of adhering tissues. The bone ends were removed and the marrow cavities were flushed by slowly injecting a media with a 26-gauge needle. The osteoblastic cells and bone marrow cells collected were washed and used in the co-culture. Osteoclasts were prepared from a co-culture system as previously described[7]. The osteoblastic cells (1.0×10^4 cells/well) were co-cultured with bone marrow cells (2.0×10^7 cells/well) in α-MEM containing 10% FBS in 96-well plates (Corning Inc.). The culture volume was made up to 200 μl per well with α-MEM supplemented with 10% FBS in the presence of 10^{-8} M $1\alpha,25(OH)_2D_3$ (Biomol) and 10^{-6} M PGE_2, with or without a sample. All cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2 in air. Three-quarter of medium was changed after co-culture for 3 days. After the cultivation, the adherent cells were fixed with 10% formaldehyde in 10 mM phosphate-buffered saline (pH 7.4) for 20 min. After being treated with 95% ethanol for 1 min, the well surface was dried and treated with the TRAP staining solution [0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate (Sigma chemical Co.), and 1 mg/ml fast red violet LB salt (Sigma chemical Co.)] for 30 min.

The TRAP-positive multinucleated cells were then counted under a microscope. Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma chemical Co.) assay. After the culture, cells were treated with 1 mg/ ml MTT for 2 hours, then precipitated dye was solubilized into dimethylsulfoxide, and the absorbance was measured at 570 nm.

3.5. Statistical Analysis

Data thus collected were analyzed statistically using Student's *t*-test to determine significant difference in the data among the groups. *P* values less than 0.05 were considered significant. The values are expressed as mean ± SE.

Acknowledgment

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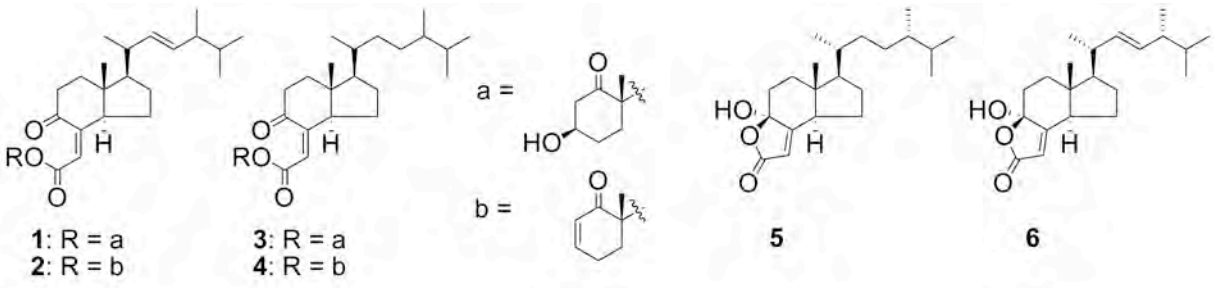
Figure Legends

Figure 1. 2D NMR Correlations of chaxine B (**1**).

Figure 2. Inhibition of osteoclast formation by **1** and **2**. Closed and open columns indicate cell viability and osteoclast formation, respectively. TRAP-positive multinucleated cells that had more than three nuclei were counted. Cell viability was determined by MTT assay. Data are the mean ± SE of two cultures (*p<0.05 vs control using Student's t-test).



1: R = a
2: R = b
3: R = a
4: R = b

Scheme 1. Choi et al.

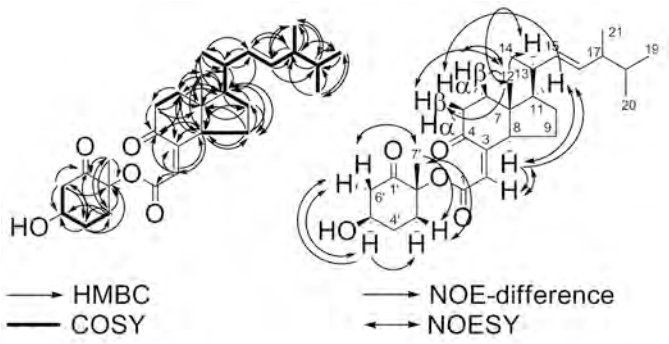


Figure 1. Choi et al.

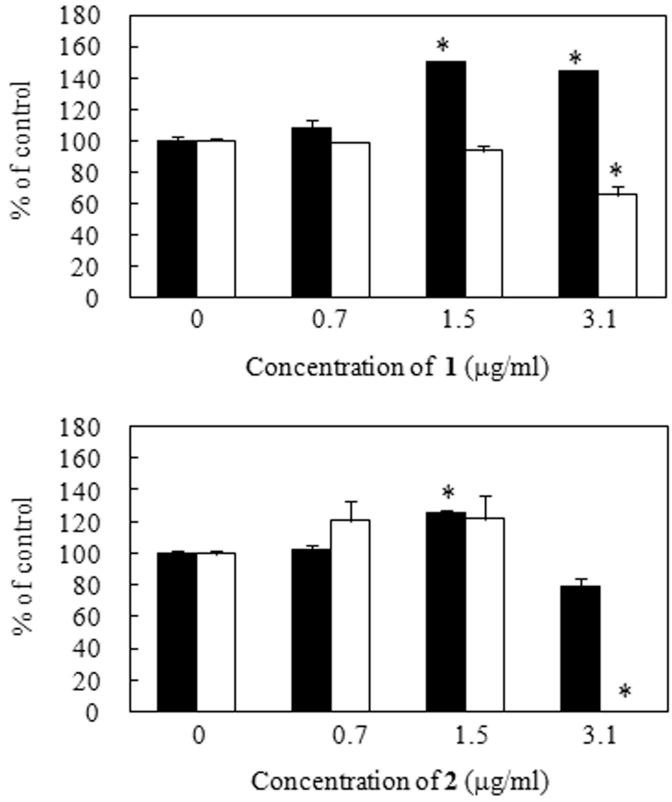


Figure 2. Choi et al.

Table 1 ^{1}H and ^{13}C NMR data for **1**-**4** (in $CDCl_3$)

Position	chaxine B (**1**)		chaxine C (**2**)		chaxine D (**3**)		chaxine E (**4**)	
	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C
1	—	165.3	—	164.2	—	165.3	—	164.2
2	5.55 (d, 2.4)	117.4	5.66 (d, 1.5)	117.5	5.56 (d, 2.4)	117.3	5.66 (d, 1.5)	117.4
3	—	155.9	—	156.2	—	156.0	—	156.3
4	—	203.5	—	204.6	—	203.5	—	204.6
5α	2.48 (m)	38.4	2.43 (m)	39.0	2.46 (m)	38.8	2.43 (m)	39.0
5β	2.67 (m)		2.70 (ddd, 13.4, 7.0, 7.3)		2.67 (m)		2.70 (ddd, 13.4, 7.3, 7.9)	
6α	1.68 (ddd, 13.1, 13.1, 5.2)	37.7	1.65 (ddd, 13.4, 12.8, 5.5)	38.0	1.68 (m)	37.8	1.64 (m)	38.1
6β	2.20 (m)		2.18 (m)		2.21 (m)		2.21 (m)	
7	—	46.2	—	46.5	—	46.3	—	46.6
8	2.50 (m)	57.5	2.44 (m)	57.8	2.49 (m)	57.4	2.44 (m)	57.8
9	1.47 (m), 1.59 (m)	22.0	1.46 (m), 1.53 (m)	21.8	1.48 (m), 1.59 (m)	22.0	1.46 (m), 1.53 (m)	21.9
10	1.47 (m), 1.89 (m)	28.9	1.45 (m), 1.86 (m)	29.0	1.48 (m), 1.89 (m)	28.7	1.46 (m), 2.02 (m)	28.7
11	1.40 (m)	55.4	1.38 (dd, 18.3, 9.0)	55.3	1.40 (m)	55.4	1.37 (m)	55.4
12	0.86 (s)	12.1	0.85 (s)	12.1	0.84 (s)	11.8	0.84 (s)	10.5
13	2.07 (m)	40.1	2.06 (m)	40.1	1.37 (m)	36.1	1.39 (m)	36.1
14	1.01 (d, 6.7)	21.0	1.00 (d, 6.4)	21.0	0.93 (d, 6.1)	18.8	0.91 (d, 6.4)	18.8
15	5.13 (dd, 15.3, 8.5)	134.5	5.12 (dd, 15.3, 8.5)	134.6	0.96 (m), 1.39 (m)	33.3	0.96 (m), 1.40 (m)	33.4
16	5.25 (dd, 15.3, 7.9)	133.0	5.23 (dd, 15.3, 7.6)	132.9	0.95 (m), 1.37 (m)	30.5	0.95 (m), 1.39 (m)	30.5
17	1.84 (m)	42.8	1.84 (m)	42.8	1.23 (m)	39.0	1.23 (m)	39.0
18	1.46 (m)	33.0	1.45 (m)	33.0	1.57 (m)	31.5	1.57 (m)	31.5
19	0.82 (d, 7.0)	19.9	0.82 (d, 7.6)	19.6	0.76 (d, 6.7)	17.6	0.76 (d, 7.0)	17.6
20	0.80 (d, 7.0)	19.6	0.80 (d, 7.6)	19.9	0.84 (d, 6.7)	20.5	0.84 (d, 6.4)	20.5
21	0.90 (d, 7.0)	17.6	0.90 (d, 6.7)	17.6	0.77 (d, 6.7)	15.4	0.77 (d, 6.7)	15.4
1′	—	204.5	—	196.7	—	204.5	—	196.9
2′	—	82.6	—	81.2	—	82.6	—	81.2
3′	1.53 (m), 2.32 (m)	33.2	2.01 (m)	32.1	1.53 (m), 2.33 (m)	33.1	2.01 (m)	32.1
			2.91 (ddd, 12.1, 6.1, 5.7)				2.91 (ddd, 12.1, 6.1, 5.7)	
4′	1.90 (m), 1.95 (m)	29.6	2.40 (m), 2.45 (m)	24.7	1.90 (m), 1.95 (m)	29.5	2.40 (m), 2.45 (m)	24.8
5′	3.93 (m)	70.1	6.86 (m)	148.1	3.94 (m)	70.1	6.87 (m)	148.8
6′	2.67 (m)	47.7	6.01 (d, 10.5)	128.1	2.67 (m)	47.7	6.02 (d, 11.9)	128.1
	2.86 (dd, 13.1, 8.9)				2.86 (dd, 13.1, 8.9)			
7′	1.43 (s)	20.3	1.46 (s)	21.9	1.43 (s)	20.3	1.46 (s)	21.9